https://doi.org/10.26651/allelo.j/2021-51-1-1310 0971-4693/94 Euro 20.00
Allelopathy Journal 52 (1):103-118 (January, 2021) *International Allelopathy Foundation* 2021
Tables: 2, Figs : 4

Effects of rhizome extracts from invasive knotweed species *Fallopia japonica* and F. ×*bohemica* on radish seed germination and root growth of seedlings

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(Received in revised form: November 23, 2020)

ABSTRACT

Japanese knotweed (*Fallopia japonica*) (Houtt.) Ronse Decr. and Bohemian knotweed (*F.* ×*bohemica*) (Chrtek and Chrtková) J. P. Bailey are invasive plants in Slovenia. We studied the effects of aqueous extracts [0.5 %, 1 %, 2 %, 5 % and 10 % (w/v)] from rhizomes of *F. japonica* and *F.* ×*bohemica* on the seeds of radish (*Raphanus sativus*) and examined the morphological and biochemical changes during germination and early growth of seedlings. Germination and early growth of radish were monitored 3, 5 and 7 days after treatment. Extracts of these two knotweed taxa delayed seed germination and strongly reduced the length of the primary root, but had less effect on shoot growth. These extracts triggered stress-induced morphogenic responses in the treated radish seedlings, stimulating the formation of lateral roots at low concentrations and causing inhibition at high concentrations. The extract concentration and not the knotweed taxon influenced the biochemical markers of oxidative stress in the radish. Total antioxidative capacity was increased in treated radish seedlings. The extract of *F. japonica* had a greater impact on the radish morphology than that of *F.* ×*bohemica*, with similar influences on the biochemical parameters. High pressure liquid chromatography identified emodin, resveratrol, catechin and epicatechin in the rhizomes of both knotweed species. Their contents were species dependent.

Key words: Bohemian knotweed, early growth, extract, *Fallopia japonica*, germination, high pressure liquid chromatography, Japanese knotweed, oxidative stress, phytotoxicity, radish, *Raphanus sativus*, rhizome

INTRODUCTION

Some plant species have spread around the world, became dominant in their new habitat and adversely affected the native plant communities (8). One such most aggressive invasive alien plant species in Europe and North America is the perennial shrub *Fallopia japonica* var. *japonica* (Houtt.) Ronse Decr. (Japanese knotweed) (Fig. 1A,B). It originates in East Asia and was introduced as an ornamental plant into Europe in the 19th century. Due to its competitive nature, vegetative reproduction and rapid growth, it has become dominant over native plants, reducing the biodiversity (2). It is listed among the top 100

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most invasive taxa worldwide (21). In Europe, *F. japonica* can hybridise with *Fallopia sachallinensis* (F. Schmidt) Ronse Decr. (giant knotweed), becoming even more invasive and genetically variable *Fallopia* ×*bohemica* (Chrtek and Chrtková) J. P. Bailey (Bohemian knotweed) (Fig. 1C,D) (2,30). However, relatively few data are available for this taxon, hence, new analysies is needed.

Knotweeds are causing severe ecological problems in Slovenia, especially *F. japonica* and *F.* ×*bohemica*, are most frequently found (32). The most recognizable characteristics for their identification are the size and shape of the fully developed leaves and the trichome type on the abaxial leaf surface. The leaves of *F. japonica* are 10 cm to 15 cm long and have a truncate base and single-celled blunt trichomes, whereas the leaves of *F.* ×*bohemica* are larger (up to 25 cm long) and have a weak to moderately cordate base and two-to-three-celled pointed trichomes (2).

Figure 1. *Fallopia japonica* (A,B) and *F.* ×*bohemica* (C,D) whole plants and closer view of leaves and flowers. Photo: S. Strgulc Krajšek.

These rapid growing and robust perennial herbs colonise degraded and disturbed habitats along riverbanks, roads and railways (31). They spread quickly through vegetative reproduction, with an extensive rhizome system and stem fragments from which shoots rapidly emerge early in spring. Their above-ground shoots grow up to 3 m height (2), produce large amount of biomass (14) and the dense canopy shade strongly inhibits growth of nearby plants (22).

Although rapid vegetative growth is the major reason for knotweed dominance over the native flora (4), allelopathy also appears to play role in their invasion (23). According to the ‘Novel weapon hypothesis of invasiveness’, invasive alien plant species can release allelopathic compounds into the soil, which suppresses the growth and development of nearby plants (8). In knotweeds, allelopathic compounds mainly accumulate in the underground rhizome (9,14,37) and many biologically active

compounds [resveratrol, (-)-catechin, (-)-epicatechin, piceid, resveratroloside, emodin, piceatannol glucoside and emodin-8-O-glucoside] have been identified in knotweeds (12).

Allelopathic compounds induce production of reactive oxygen species in target plants, and an imbalance in their antioxidative system. This causes oxidative stress (5) and results in lipid peroxidation. The activities of antioxidative enzymes (catalase and peroxidase (11) and the levels of non-enzymatic antioxidants changes in plants during stress, and thus represent a marker of plant physiological status (28). At the organism level, the most common allelopathic effects are inhibition of germination and suppression of growth. Some of these effects occur after the application of knotweed secondary compounds (13,34), knotweed extracts (12,22,36) and dried knotweed material (12,33) to other plants, and when knotweeds were grown together with other plants (23,30).

The bioactivity of methanol extracts (13,34,35) and 70 % aqueous acetone extracts (17) of knotweeds had been investigated. In this study, aqueous extracts were used to test conditions that are more similar to the rhizosphere in nature. Besides, we also measured the content of four most important allelochemicals in aqueous and methanol extracts, because phenolic compounds are better dissolved in organic solvents.

In our previous study, the aqueous leaf extracts of *F. japonica* and *F. ×bohemica* strongly inhibited the roots length of radish seedlings (12). As rhizomes have more allelopathic compounds than leaves (9,14), hence, we examined the rhizome extracts. Thus, the phenolic profiles of these *F. japonica* and *F. ×bohemica* extracts were compared, and their allelopathic effects were evaluated on radish (*Raphanus sativus* L.). Brassicaceae family plants are frequently used in allelopathic studies (12,13,22,33) due to their sensitivity and rapid growth, and thus radish was selected as the test specie. The duration of many allelopathic studies have been either ≤ 3 days (13,36) or >14 days (23,30,34). In this study, we determined the morphological and biochemical changes at 3, 5 and 7 days in radish seed germination and seedling growth.

Therefore, based on the known biological activities of knotweed, we tested knotweed extracts for effects on germination and early growth of radish. This study aimed to evaluate the phytotoxic effects of knotweed extracts to assess their potential as bioherbicides. For this we determine, whether: (a) the phenolic profiles of *F. japonica* and *F. ×bohemica* rhizomes differ; (b) their aqueous extracts have different phytotoxic effects on radish; (c) the roots and shoots of the treated radish seedlings have different susceptibilities to these aqueous extracts and (d) effects of aqueous extract concentrations on the morphological and biochemical changes in the treated radish seedlings.

MATERIALS AND METHODS

Plant material

The rhizomes of Japanese knotweed (*Fallopia japonica* var. *japonica* (Houtt.) Ronse Decr.) and Bohemian knotweed (*Fallopia ×bohemica* (Chrtek and Chrtková) J.P. Bailey) were collected in Ljubljana, Slovenia (46° 2′ 33.98″ N, 14° 27′ 0.91″ E; 46° 3′ 0.3″ N, 14° 28′ 44″ E; respectively) in March and April 2016. The rhizomes were rinsed with tap water to remove adhering soil, then dried, lyophilised (5 days, -98 °C, 0.003 mbar), ground to powder and stored in dark at room temperature prior to high pressure liquid chromatography (HPLC) analysis and extract preparation.

Chemicals

All chemicals for HPLC analyses were of analytical grade. Acetonitrile (gradient grade), phosphoric acid and acetic acid were from Merck (Darmstadt, Germany), methanol from VWR Chemicals (Radnor, USA). MilliQ water (18.2 MΩ) was used for the preparation of all aqueous solutions. The standards of catechin, epicatechin, emodin and resveratrol were from Sigma-Aldrich (St. Louis, MO, USA). Potassium phosphate buffer was prepared from dipotassium hydrogen phosphate (K_2HPO_4) and potassium hydrogen phosphate (KH_2PO_4), both from Acros Organics (New Jersey, USA). Bicinchoninic acid protein assay kits were from Novagen (San Diego, USA). Thiobarbituric acid was from Fluka (Steinheim, Germany), and trichloroacetic acid was from Acros Organics (New Jersey, USA). Guaiacol was from Sigma-Aldrich (St. Louis, MO, USA) and hydrogen peroxide (H_2O_2) was from Belinka, Perkemija (Ljubljana, Slovenia). 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Sigma-Aldrich (Steinheim, Germany), and methanol and acetone were from Merck (Darmstadt, Germany).

HPLC analysis

Standard solutions of emodin, resveratrol, catechin and epicatechin (all at 1 mg mL^{-1} concentration) were individually prepared in 33 % methanol and stored at -20 °C. The solutions were diluted to the final concentration of 10 µg mL^{-1} prior to HPLC analysis.

To obtain aqueous and methanol extracts, the ground rhizome material from *F. japonica* and *F.* ×*bohemica* (2 g) was resuspended in distilled water (20 ml) or 33 % methanol (20 ml), respectively and shaken (175 rpm) for at least 24 h at room temperature in dark. The suspensions were then filtered (Grade 520A, Whatman, Maidstone, UK) and stored at 4 °C. The extracts were filtered again (Millex GS, pore 0.22 um) prior to HPLC analysis.

For the resveratrol and emodin content in the samples, HPLC analyses were performed with Luna Omega Polar 5 µm C18 250 x 4,6 mm (Phenomex) column and Waters 2695 Separation Module and 2996 PDA detector using a modified method by Cheng et al. (10). The mobile phase consisted of acetonitrile (gradient) (A) and 1.5 % phosphoric acid in ddH_2O (B). The gradient elution proceeded as follows: 0-10 min isocratic elution (44 % A), 10-25 min linear gradient elution (44-82 % A). The injection volume was 25 µL and the flow rate 1 mL min^{-1} at column temperature of 30 °C. The retention times were 4.8 min for resveratrol and 20.5 min for emodin. The detection was performed at 320 nm for resveratrol and 435 nm for emodin.

The catechin and epicatechin contents were quantified as per Gürbüz *et al.* (18). The analytical system was the same as above except for the detector, since a fluorimetric detector (Waters 2475) was used. The mobile phase consisted of acetonitrile (gradient) (A) and 5 % acetic acid in ddH_2O (B). The gradient elution was as under: 0-10 min isocratic elution (9 % A), 10-11 min linear gradient elution (9-25 % A), 11-20 min isocratic elution (25 % A). The injection volume was 10 µL and the flow rate 1 mL min^{-1} at column temperature of 30 °C. The retention times were 7.5 min for catechin and 11.4 min for epicatechin. The detection was performed at wavelengths (k_{Ex}/k_{Em}) 280/315 nm.

Aqueous extracts of *Fallopia* rhizomes

The ground rhizome material (5 g) was re-suspended in distilled water (50 mL), shaken (175 rpm) for 24 h at room temperature and was then vacuum filtered (Grade 520A, Whatman, Maidstone, UK) to get the final extract with 10 % (w/v) concentration. It was diluted with distilled water to prepare 0.5, 1, 2 and 5 % concentrations. The extracts electrical conductivity and pH were measured by multimeter PCD 60 (Eutech, Singapore) and stored at 4 °C.

Bioassay

The experimental treatments consisted of two factors, (i). Donor species: 2 (*Falloppia japonica* and *Fallopia ×bohemica*) and (ii). Aqueous extract concentrations: 5 (0.5, 1.0, 2.0, 5.0 and 10.0 %). The treatments were replicated thrice in complete randomised design. The radish seeds were germinated under laboratory conditions (22 ± 2 °C, 15 h light/9h dark). Fifty seeds of radish (*Raphanus sativus* L. cv. Saxa 2) were placed on a layer of filter paper in a Petri dish (14 cm dia). The control seeds were watered with 10 mL distilled water, while knotweed extract (10 mL) was applied to the experimental seeds. The seedlings were watered after 3 and 5 days with 3 mL distilled water and the experiment lasted 7 days. On days 3, 5 and 7, the germinating seeds were counted to determine the germination rate, and photographed. The length of the primary root and shoot and the number of lateral roots were determined after 3, 5 and 7 days with the digital photographs using the ImageJ 1.× software (29). After 7 days, the roots and shoots were separated using a scalpel, weighed, frozen in liquid nitrogen and stored at -20 °C, prior to the biochemical analyses.

Biochemical analyses

Frozen samples of 100 mg radish roots or shoots were homogenised in 1.5 mL potassium phosphate buffer (100 mM, pH 7.0) and centrifuged at 15339× *g* for 20 min at 4 °C. The supernatants were used for spectrophotometric analyses (UV-1800; Shimadzu, Kyoto, Japan) of lipid peroxidation, protein concentrations (bicinchoninic acid protein assay kits), specific enzyme activities (guaiacol peroxidase, catalase) and total antioxidative capacity, were determined as per Dolenc Koce and Šoln (12) as under.

(i). Lipid peroxidation: It was measured as the malondialdehyde content. The supernatant (200 µL) was added to 800 µL acetic reagent of 0.5 % (w/v) thiobarbituric acid in 20 % (w/v) trichloroacetic acid, incubated for 30 min at 95 °C, and then chilled on ice to stop the reaction. The malondialdehyde content was determined spectrophotometrically at 532 nm and 600 nm.

(ii). Enzymes activity: The activity of enzyme guaiacol peroxidase (EC 1.11.1.7) was measured spectrophotometrically at 470 nm (e = 26.6 µM $min^{-1}mg^{-1}$) using 20 µL supernatant and 980 µL reaction mixture containing potassium phosphate buffer (50 mM, pH 7.0), 1 % guaiacol and 10 mM H_2O_2. The activity of enzyme catalase (EC 1.11.1.6) was measured spectrophotometrically at 240 nm (e = 40 µM $min^{-1}mg^{-1}$) using 50 µL supernatant and 950 µL reaction mixture containing potassium phosphate buffer (50 mM, pH 7.0) and 10 mM H_2O_2.

(iii). Total antioxidant capacity: It was measured spectrophotometrically at 515 nm as the content of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), as described by Sánchez-Moreno et al. (28), and slightly modified (12). Radish roots or shoots (100 mg) were homogenised in 2 mL methanol and centrifuged at 15339× *g* for 5 min at 4 °C. This supernatant (30 µL) was added to 2 mL 120 µM DPPH and incubated in the dark for 15 min. To calculate the total antioxidant capacity, a calibration curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used, with the following concentrations: 0.2, 0.4, 0.6, 0.8, 1.5 and 2 mM.

(iv). Photosynthetic pigments: The contents of chlorophyll *a*, chlorophyll *b* and carotenoids were determined in radish shoots. The frozen plant material (400 mg) was homogenised in 6 mL acetone and centrifuged at 3287× *g* for 5 min at 4 °C, the supernatant was measured spectrophotometrically at 645 nm (chlorophyll *a*), 662 nm (chlorophyll *b*) and 470 nm (carotenoids) (6).

Statistical analysis

Means and standard errors were calculated for each measured parameter. Means among control and treatment groups were compared using one-way ANOVA, and Holm-Sidak *post-hoc* test was used when differences were statistically significant. Two-way ANOVA was used to compare the effects of knotweed taxa, extract concentration and their interaction (Microsoft Excel with XL Toolbox 7.2.13). Differences in the content of four active compounds in rhizomes between the two knotweed species and the extraction solvents were evaluated by one-way ANOVA and Holm's *post-hoc* adjustment using statistical software R v. 4.0.2 (27) and Library Agricolae 1.3-3. The level of statistical significance was set for $P < 0.05$.

RESULTS AND DISCUSSION

We found that aqueous extracts from *F. japonica* and *F. ×bohemica* rhizomes had strong effects on radish seeds, germination and seedlings growth.

HPLC analysis of *F. japonica* and *F. ×bohemica* rhizomes

The rhizomes of both knotweed species contained all four tested allelochemicals [resveratrol, emodin, catechin and epicatechin] in concentrations ranging from 0.42 mg mL^{-1} to 66.58 mg mL^{-1} and their content was extraction and taxon dependent (Table 1). Methanol extraction yielded up to 10-times higher concentrations of the allelochemicals than aqueous extraction. The rhizomes of *F. japonica* contained more resveratrol than *F. ×bohemica*, while *F. ×bohemica* contained more emodin, catechin and epicatechin than *F. japonica.*

(i). Resveratrol: Its concentrations ranged from 0.54 mg mL^{-1} to 8.45 mg mL^{-1}. Methanol and aqueous extracts of *F. japonica* contained 1.5 and 2.8-times more resveratrol than *F. ×bohemica*, respectively (Table 1). In previous studies, there was 10-folds difference between the two knotweed species, when 50 % methanol was used for extraction (9).

Table 1. Concentrations of emodin, resveratrol, catechin and epicatechin in rhizomes of *F. japonica* and *F. ×bohemica.*

Extract	Concentration (mg mL^{-1})			
	Emodin	Resveratrol	Catechin	Epicatechin
		Fallopia japonica		
Methanol extract	2.36 ± 0.01^{b}	8.45 ± 0.003^{a}	3.96 ± 0.02^{c}	10.42 ± 0.05^{b}
Aqueous extract	0.73 ± 0.01^{d}	1.54 ± 0.004^{c}	0.42 ± 0.003^{d}	1.99 ± 0.01^{d}
		Fallopia ×bohemica		
Methanol extract	4.39 ± 0.01^{a}	5.77 ± 0.13^{b}	19.25 ± 0.03^{a}	66.58 ± 0.16^{a}
Aqueous extract	1.34 ± 0.01^{c}	0.54 ± 0.02^{d}	10.95 ± 0.13^{b}	36.28 ± 0.15^{c}

Data are means ± SE (n=3). Different letters indicate statistically significant differences between knotweed species and extraction solvents for each compound (one-way ANOVA, $P < 0.05$).

When comparing the phenol content in three invasive knotweeds, *F. japonica* contained more resveratrol, resveratroloside than the hybrid *F. ×bohemica* and *F. sachalinensis* (14). The phenolics profile in rhizomes of *F. ×bohemica* was more similar to *F. japonica* than to *F. sachalinensis*, as the rhizomes of *F. sachalinensis* do not contain resveratrol (25).

(ii). Emodin: Its concentrations in knotweed rhizomes ranged from 0.73 mg mL^{-1} to 4.4 mg mL^{-1}, and were higher in *F. ×bohemica* (Table 1). On the other hand, Frantík *et al.* (14) reported no differences in emodin concentration in knotweed rhizomes in first two years, while *F. japonica* rhizomes contained more than twice the emodin concentration in *F. ×bohemica* rhizomes after 3-years cultivation. Besides the plant age, growth period (9,14) and geographical area (9,13) influences the chemical composition of the plant material. The rhizomes of native *F. japonica* from China contained more emodin than the invasive populations in Canada, while the contents of resveratrol, polydatin and physcion were high but similar (9). Therefore, the chemical composition of invasive knotweeds growing in Slovenia may differ from knotweeds growing elsewhere due to different environmental and growth conditions.

(iii). Catechin and epicatechin: Their concentrations were highest (up to 66.58 mg mL^{-1}) in rhizomes of *F. ×bohemica* (Table 1). Previous studies showed that the spring sprouts of *F. japonica* contained more catechin than *F. ×bohemica*, while epicatechin levels were similar in both taxa (35). Again, the differences can be attributed to different conditions at collection site of knotweeds and their developmental stage.

In addition to environmental and growth conditions, the extraction solvent also influences the phenols contents. Our results showed that extraction with 33 % methanol solution yielded 3 to even 10 times more compounds than extraction with distilled water (Table 1), as phenols are more soluble in organic solvents. For this reason, previous studies used methanol (9,13,25,35), ethanol (14) or acetone extracts (17). In this study, however, we tested the allelopathic activity of aqueous extracts because they are closer to the natural condition in rhizosphere, where allelochemicals are released in the soil from roots or leaf litter. We showed that aqueous extracts also contained significant concentration of phenols, and we used them in germination and growth studies.

Germination and seedling growth of radish

The rhizome extracts of *F. japonica* and *F. ×bohemica* inhibited the radish germination only at the beginning, while on days 5 and 7 the seed germination rates of treated and control were similar (Figure 2). The extract concentrations and the knotweed taxa were the factors that significantly (P = 0.031) reduced the seed germination by 5 % and 9 % respectively at 10 % extracts of F. *japonica* and *F. ×bohemica* on day 3.

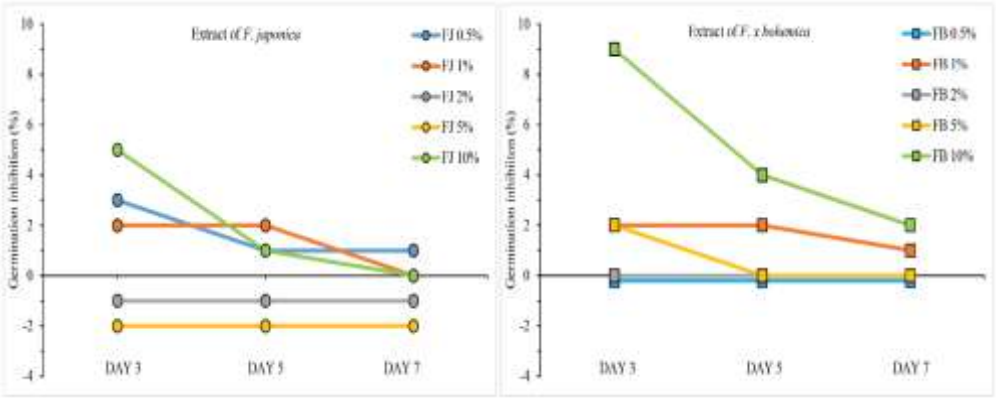

Figure 2. Effects of extracts from rhizomes of *F. japonica* and *F. ×bohemica* on seeds germination of radish seeds. The results show inhibition of seeds germination over the control.

These inhibitions are similar to those reported for the rhizome extracts of three invasive knotweeds that reduced seeds germination of white mustard (*Leucosinapis alba*) (36) and radish when exposed to allelopathic compounds [resveratrol, (-)-epicatechin, emodin and polydatin] from knotweeds (34). On the other hand, knotweed leaves also strongly inhibits the radish seeds germination, with higher phytotoxicity of *F. ×bohemica* extracts (22). Higher phytotoxic effect of *F. ×bohemica* corresponds to previous studies (2,22) and field observations in Slovenia that this specie is stronger competitor than its congeners (31). The variability in seeds germination also depends on the recipient test crop. Indeed, seeds of crops (*Leucosinapis alba* and *Brassica napus*) were more sensitive to knotweed material in soil than the weeds seeds viz., *Chenopodium album* and *Echinochloa crus-galli* (33). The *Urtica dioica* and *Calamagrostis epigejos* were more sensitive to knotweeds extracts than *Lepidium sativum* (22). These differences indicate that the source of plant material and its chemical composition can be correlated to the effects observed in the test species. Later on days 5 and 7, the germination in extracts concentrations of both *Fallopia* species was lower and similar to the control.

(i). Root growth: The subsequent growth of radish seedlings was influenced by *Fallopia* extracts. The root growth was drastically reduced, as the roots growth of the treated seedlings were less than half of controls (Fig. 3). Roots are the primary target of many allelochemicals, especially during the early stages of growth and development, when metabolic activity is high and they are more sensitive to environmental stresses (1). This study showed that extracts of *F. japonica* and *F. ×bohemica* had significant (P < 0.001)

concentration-dependent inhibitory effects on the length of radish roots after 3, 5 and 7 days. The seedling roots exposed to the knotweed extracts were up to 65 % smaller than control seedlings. The reduced root growth in this study is similar to Tucker Serniak (34), who reported that radish exposed to allelopathic compounds of Japanese knotweed had shorter roots. Moravcová *et al.* (22), reported that *F.* ×*bohemica* extract had stronger phytotoxic potential than *F. japonica.* Contrarily our results were opposite. The effects of *F. japonica* and *F.* ×*bohemica* extracts differed only on day 3, when the roots exposed to *F. japonica* extract were 34 % shorter than those exposed to *F.* ×*bohemica* extract ($P < 0.001$). On days 5 and 7, there were no differences in the effects of extracts of these two knotweed taxa (Fig. 3). From these results, we conclude that although the extracts had different chemical composition, their effects on radish roots were similar.

(ii). Shoot growth: In contrast to roots, the shoot length of radish was less influenced by the *Fallopia* extracts (Fig. 2). There was only a transient response at highest concentrations, where the shoot length on 3rd and 5th days was up to 43 % lower than control seedlings. The shoots were less affected by *Fallopia* extracts, as they were not in direct contact with extracts. A similar response of radish exposed to different secondary metabolites of knotweeds was reported (34). However, we infer that reduced shoot growth was due to delayed germination rather than to negative effects of extracts.

In addition to the inhibitory effects, extracts with lower concentrations also had some stimulating effects as observed on day 5 (Fig. 3). The seedlings exposed to 1 % extracts had up to 32 % longer roots and shoots than control ($P < 0.001$). This phenomenon is called 'hormesis', when an otherwise toxic substance can have positive effects at low doses. Other compounds in extracts, such as microelements, ions and salts, can also promote the growth of seedlings (3). Therefore, not only inhibitory but also stimulatory compounds should be analysed for these extracts in the future. In addition to biologically active compounds, the chemical and physical properties of the extracts can also influence their efficacy. The pH of both *Fallopia* extracts was acidic (4.46 ± 0.30 for *F. japonica*, 3.24 ± 0.09 for *F.* ×*bohemica*) and not correlated with the extract concentrations. Since phenolic compounds are influenced by the basic pH and are more stable under acidic conditions, as shown for resveratrol (38) and some others (15). We conclude that pH had no significant influence on the biological activity of allelochemicals in the extracts.

The *Fallopia* extracts moderately affected the formation of lateral roots, as 20 % of treated seedlings did not develop any lateral roots (Fig. 4). The reduction was significantly influenced by the extract concentration on days 3, 5 and 7 ($P = 0.041$, $P < 0.001$, $P = 0.003$, respectively). The treated seedlings that developed lateral roots showed variable effects of extract concentration (Fig. 4); lower concentrations (0.5 %) stimulated the formation of lateral roots up to 25 % higher root number per seedling than in control, while higher concentrations (5 %, 10 %) reduced the formation of lateral roots up to 54 %. The extract concentration had significant impact on the number of lateral roots on days 3, 5 and 7 ($P = 0.017$, $P < 0.001$, $P = 0.003$, respectively). When plants are under stress, they respond by modifying their root morphology. This acclimation strategy is known as the 'stress-induced morphogenic response'. It enables plants to survive exposure to sub-lethal doses of toxic substances (26), such as heavy metals (24), or other stressors [UV-B

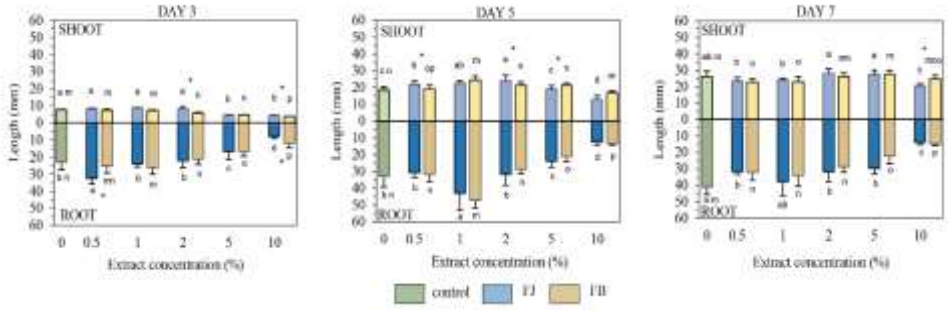

Figure 3. Effects of extracts from rhizomes of *F. japonica* (FJ) and *F. ×bohemica* (FB) on growth of radish shoots (upwards)-and roots (downwards) 3, 5 and 7 days after treatment. Data are means ± SE (n = 150). Different letters indicate statistically significant differences between control seedlings and seedlings treated with FJ (a-d) and FB (m-r). *, *P* <0.05, between knotweed extracts of the same concentration (one-way ANOVA).

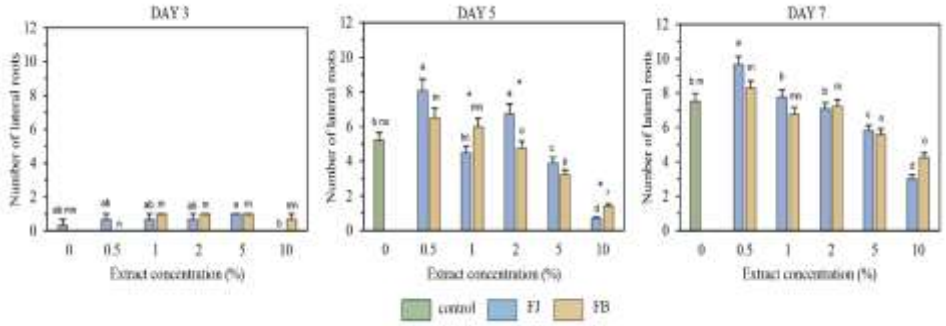

Figure 4. Effects of extracts from rhizomes of F. japonica (FJ) and F. ×bohemica (FB) on development of radish lateral roots 3, 5 and 7 days after treatment. Data are means ±SE (n = 150). Different letters indicate statistically significant differences between control seedlings and seedlings treated with FJ (a-d) and FB (m-r). *, P <0.05, between knotweeds extracts of the same concentration (one-way ANOVA).

radiation (19), hypoxia (16) and phosphate starvation (20)], or where these are part of phytotoxic response as reported for cucumber seedlings treated with allelochemicals from *Secale cereale* (7).

Biochemical analysis of radish seedlings

The response of plants to stress can also be associated with the production of reactive oxygen species (26), and therefore, biochemical markers of oxidative stress have been analyzed for radish seedlings treated with knotweed extracts (Table 2). In the roots, the activity of the antioxidative enzyme guaiacol peroxidase was lower in the treated

Table 2. Effects of extracts from rhizomes of *F. japonica* (FJ) and *F. ×bohemica* (FB) on biochemical parameters in radish root and shoot after 7 days of treatment

	F. japonica						*F. ×bohemica*				
Parameter	0%	0.5%	1%	2%	5%	10%	0.5%	1%	2%	5%	10%
Roots											
CAT (μM min^{-1} mg^{-1})	0.01±0.00	0.07±0.01*	0.03±0.01	0.09±0.06	0.07±0.06	0.01±0.00	0.08±0.05	0.03±0.01	0.07±0.03	0.05±0.02	0.04±0.03
G-POD (nM min^{-1} mg^{-1})	1.41±0.24	0.93±0.64	0.80±0.11	0.18±0.98	1.04±0.42	0.17±0.09	0.19±0.08	0.26±0.01	0.68±0.30	2.45±0.95	0.67±0.14
MDA (nM/g)	10.84±3.16	9.97±1.45	12.68±1.06	13.32±3.06	10.13±0.77	12.93±0.47	13.03±1.61	13.03±0.39	12.58±0.77	11.26±0.03	10.77±1.29
TAC (mM)	0.84±0.01	0.88±0.00*	0.86±0.03	0.92±0.03	0.85±0.01	0.94±0.04	0.89±0.02	0.96±0.03*	0.93±0.02*	0.88±0.03	0.94±0.02*
Shoots											
CAT (μM min^{-1} mg^{-1})	10.07±4.29	11.54±0.55	9.71±1.86	7.47±1.49	4.66±0.46	5.43±3.25	11.28±3.02	13.26±3.25	12.73±3.32	11.67±1.07	11.09±1.66
G-POD (nM min^{-1} mg^{-1})	0.17±0.06	0.17±0.01	0.18±0.03	0.18±0.05	0.09±0.01	0.17±0.07	0.20±0.04	0.33±0.19	0.23±0.00	0.23±0.01	0.12±0.07
MDA (nM/g)	5.19±1.00	9.94±1.10	6.10±0.55	6.65±0.52	10.55±1.90	14.03±2.23*	15.07±2.42*	12.90±1.29*	11.29±3.23	7.90±1.19	10.10±2.48
TAC (mM)	1.44±0.04	1.50±0.01	1.44±0.03	1.55±0.05	1.55±0.06	1.53±0.01	1.50±0.02	1.53±0.07	1.48±0.05	1.45±0.02	1.56±0.01
Chl *a* (mg/g)	0.18±0.02	0.22±0.03	0.19±0.01	0.20±0.03	0.17±0.01	0.24±0.02	0.21±0.01	0.25±0.04	0.19±0.02	0.17±0.02	0.20±0.02
Chl *b* (mg/g)	0.06±0.01	0.09±0.03	0.06±0.00	0.07±0.02	0.05±0.00	0.09±0.02	0.07±0.003	0.09±0.02	0.06±0.01	0.05±0.01	0.07±0.01
Carotenoids (mg/g)	0.07±0.01	0.08±0.01	0.07±0.00	0.07±0.01	0.06±0.01	0.09±0.01	0.08±0.004	0.09±0.02	0.07±0.01	0.06±0.01	0.07±0.01

Data are means ±SE (n = 3). Abbreviations: CAT (Catalase activity), G-POD (Guaiacol peroxidase activity), MDA (Malondialdehyde content), TAC (Total antioxidative capacity), Chl *a* (Chlorophyll *a*), Chl *b* (chlorophyll *b*). *, $P < 0.05$, between knotweed extracts of the same concentration (one-way ANOVA).

plants than in the control, and this effect was concentration dependent ($P = 0.015$). On the other hand, the activity of the antioxidative enzyme catalase was slightly higher in treated plants than in control, but this effect was neither concentration nor taxon dependent. Due to the variable and unspecific reaction, the catalase was not considered reliable parameter of oxidative stress. Scavenging reactive oxygen species is a complex process involving not only enzymatic but also non-enzymatic antioxidants such as ascorbate (vitamin C), α-tocopherol (vitamin E), glutathione and carotenoids (11). We used the total antioxidant capacity to estimate the level of non-enzymatic antioxidants. The results showed that the total antioxidant capacity in radish roots increased significantly in correlation with the extract concentrations ($P = 0.007$). Higher levels of non-enzymatic antioxidants provide a mechanism for the removal of reactive oxygen species and thus less cell damage. That is why in extract-treated radish roots, the lipid peroxidation (measured as malondialdehyde content), remained at control levels. Our observations support the findings of Sánchez-Moreno *et al.* (28), who reported an increased total antioxidant capacity with simultaneously reduced lipid peroxidation. Similar effects of knotweed leaf extracts were reported on radish (12). The results of oxidative and antioxidative activity in roots are consistent with the morphological observation of reduced root length after treatment with *Fallopia* extracts.

In the shoots of radish seedlings, the activity of catalase was more strongly affected by *F. japonica* extracts ($P = 0.043$). The malondialdehyde content increased both with extract concentrations and with interactions of knotweed taxa and extract concentrations ($P= 0.047$). The activity of guaiacol peroxidase, the total antioxidant capacity and the contents of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenoids) were not affected by the knotweed extracts. Most of the biochemical parameters measured in the shoots suggested that shoots in early growth stage were less affected than roots. Because the developing shoots were not in direct contact with knotweed extracts and their allelochemicals. Furthermore, during this early development, stored reserves from cotyledons were used and the shoots were less dependent on uptake by the root and its physiological condition.

CONCLUSIONS

We confirmed the phytotoxic effects of invasive *F. japonica* and *F. ×bohemica* rhizome extracts on seeds germination and seedlings growth of radish. The aqueous extracts of *F. japonica* and *F. ×bohemica* rhizomes did not inhibit but delayed the germination of radish seeds. However, they strongly suppressed the growth of seedlings roots, with moderate effects on shoots. These aqueous extracts also reduced the formation of lateral roots, indicating a stress-induced morphogenic response. The biochemical signs of oxidative stress were variable and rarely concentration dependent. The extracts of *F. japonica* inhibited the germination of radish seeds and the growth of seedlings more than *F. ×bohemica* but these differences diminished during the 7 days of experiment. From our results we conclude that although the extracts had different contents of resveratrol, emodin, catechin and epicatechin, but their effects on radish roots were similar. To understand the mechanisms of knotweeds invasiveness, further studies are required on the phytotoxicity and cellular mechanisms involved in inhibition of root growth. These

findings could enable us to estimate the potential of invasive *Fallopia* plants as agents for controlling weeds or to reduce their threat to native flora.

ACKNOWLEDGEMENTS

The authors are thankful to the anonymous reviewers for valuable comments and suggestions. We are also deeply grateful to Dr Maks Merela and his co-workers for rhizome grinding and Mr Chris Berrie for thorough reading of manuscript and English editing. This study was financially supported by the Slovenian Research Agency (Grant No. P1-0212).

The authors declare no conflict of interest.

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